The Journal of Experimental Biology 216, 4403-4414
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doi:10.1242/jeb.092809

RESEARCH ARTICLE

Exclusive localization of carbonic anhydrase in bacteriocytes of the deep-sea clam *Calyptogena okutanii* with thioautotrophic symbiotic bacteria

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SUMMARY

Deep-sea *Calyptogena* clams harbor thioautotrophic intracellular symbiotic bacteria in their gill epithelial cells. The symbiont fixes CO_2 to synthesize organic compounds. Carbonic anhydrase (CA) from the host catalyzes the reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$, and is assumed to facilitate inorganic carbon (C_i) uptake and transport to the symbiont. However, the localization of CA in gill tissue remains unknown. We therefore analyzed mRNA sequences, proteins and CA activity in *Calyptogena okutanii* using expression sequence tag, SDS-PAGE and LC-MS/MS. We found that acetazolamide-sensitive soluble CA was abundantly expressed in the gill tissue of *C. okutanii*, and the enzyme was purified by affinity chromatography. Mouse monoclonal antibodies against the CA of *C. okutanii* were used in western blot analysis and immunofluorescence staining of the gill tissues of *C. okutanii*, which showed that CA was exclusively localized in the symbiont-harboring cells (bacteriocytes) in gill epithelial cells. Western blot analysis and measurement of activity showed that CA was abundantly (26–72% of total soluble protein) detected in the gill tissues of not only *Calyptogena* clams but also deep-sea *Bathymodiolus* mussels that harbor thioautotrophic or methanotrophic symbiotic bacteria, but was not detected in a non-symbiotic mussel, *Mytilus* sp. The present study showed that CA is abundant in the gill tissues of deep-sea symbiotic bivalves and specifically localizes in the cytoplasm of bacteriocytes of *C. okutanii*. This indicates that the C_i supply process to symbionts in the vacuole (symbiosome) in bacteriocytes is essential for symbiosis.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/216/23/4403/DC1

Key words: symbiosis, chemosynthetic ecosystem, intracellular, inorganic carbon uptake, gill, bacteriocyte.

Received 20 June 2013; Accepted 26 August 2013

INTRODUCTION

Deep-sea clams belonging to the genus *Calyptogena* form dense communities on the deep-sea floor near hydrothermal vents and methane seeps (Dover, 2000; Fisher, 1990). They harbor thioautotrophic symbiotic bacteria in their gill epithelial cells and nutritionally depend on their symbionts because their digestive tracts are vestigial (Pennec et al., 1995). Genome analyses of symbionts of *Calyptogena okutanii* and *C. magnifica* revealed that they fix CO_2 *via* ribulose-1,5-bisphosphate carboxlase/oxygenase (RubisCO) and synthesize amino acids and other organic compounds, although no export system for these synthesized organic compounds to the host has been found (Kuwahara et al., 2007; Newton et al., 2007). The host may digest the symbiont and absorb the released nutrients (Kuwahara et al., 2007; Newton et al., 2007). It was shown that [^{14}C]inorganic carbon is fixed with sulfur oxidation by the *Calyptogena* symbiont (Childress et al., 1993a; Childress et al., 1991). *Calyptogena okutanii* Kojima and Ohta 1997 inhabits seep sites off Hatsushima Island, Sagami Bay, Japan, where 8–300 μmol l^{-1} hydrogen sulfide has been reported in the sediments beneath the clam community (Masuzawa et al., 1992). For CO_2 fixation by symbionts, the transport of inorganic carbon (C_i; i.e. CO_2 and HCO_3^-) from seawater to the symbionts is important. Generally, respired CO_2 in marine animal tissues is transported *via* the body fluid and finally discarded into seawater, probably through the gills (Eckert and Randall, 1978). In symbiotic bivalves harboring thioautotrophic bacteria, the C_i produced is not only eliminated but also taken up from seawater by the host animal, usually through the gill tissue. However, in equilibrium at seawater pH the majority of C_i is in the form HCO_3^- (Siegenthaler and Sarmiento, 1993). Unlike CO_2, HCO_3^- cannot pass through the cell membrane, and their interconversion ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$) rate is relatively slow (Geers and Gros, 2000). In *Calyptogena* clams, the detailed processes of uptake and transport of C_i from external seawater to symbionts are still not clearly understood.

Carbonic anhydrase (CA) is a zinc-containing enzyme catalyzing the reversible conversion between carbon dioxide and bicarbonate ion: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. CA is found in many organisms including animals, plants, archaea and bacteria (Henry, 1996; Moroney et al., 2001; Smith et al., 1999). CAs are classified into at least three families (the α-, β- and γ-CA families) based on their

amino acid sequences (Hewett-Emmett and Tashian, 1996). CAs of animals belong to the α-CA family, which is composed of cytosolic, membrane-associated, mitochondrial and secreted isoforms (Krishnamurthy et al., 2008). In animals, CA participates in a broad range of physiological processes such as acid–base homeostasis, transport of C_i and respiration (Henry, 1996). CA is important for plants and algae to take up C_i for photosynthesis (Hogetsu and Miyachi, 1979). In photosymbioses of the sea anemone *Anemonia viridis*–zooxanthellae (Furla et al., 2000) and a coral *Stylophora pistillata*–zooxanthellae (Bertucci et al., 2011), the host CA is proposed to be a key enzyme in the process of C_i uptake and its supply for symbiont photosynthesis. In *Calyptogena* clams, CA activity has been measured in the gill, foot and mantle tissue, and the highest activity was found in the gills (Kochevar and Childress, 1996). CA is therefore believed to play a major role in taking up and transporting C_i to symbionts by the host to maintain symbiosis.

The gill filament of *Calyptogena* clams consists of two major zones: (1) the external asymbiotic zone containing ciliated cells and goblet-like cells; and (2) the internal symbiotic zone containing symbiont-bearing cells (bacteriocytes) and asymbiotic cells called intermediate cells (Fiala-Médioni and Le-Pennec, 1988; Fiala-Médioni and Métivier, 1986; Morton, 1986). In bacteriocytes, the symbionts are enclosed in a vacuole called the symbiosome and isolated from the host cytosol (Fiala-Médioni et al., 1993). However, there are many unsolved questions about CA in gill tissue: which cells have CA?; which type of CA is present?; and where is the CA localized? Addressing these questions will lead to deeper insight into the important processes of symbiosis, how C_i uptake occurs in bacteriocytes in the gill tissue and how it is supplied to symbionts. In the present study, we identified CA genes by expression sequence tag (EST) analysis of the gill tissue of *C. okutanii*. Using the sequences of CA genes, CA proteins were identified and their activities were determined. We also studied CA protein localization in various tissues and cells of *C. okutanii*. Furthermore, a comparative study was undertaken to examine whether CA is generally present in chemosynthetic bivalves. The importance and role of CA in the mechanism of C_i uptake and supply in chemosynthetic symbioses are discussed.

MATERIALS AND METHODS

Animal sampling

Bivalves were collected from cold seeps and hydrothermal vent areas near Japan in 2009–2010 using the Remotely Operated Vehicle (ROV) *Hyper-Dolphin* and the Deep Submergence Research Vehicle (DSRV) *Shinkai 6500* of the Japan Agency for Marine-Earth Science and Technology (Table 1). After the bivalves had been collected onbard, blood was collected from the adductor muscle using a 10 ml syringe with a 22 gauge needle, and then blood cells were harvested by centrifugation at 250 ***g*** for 5 min at 4°C. The gill, foot and mantle tissues were immediately dissected, separated and washed several times with 0.22 μm filtered seawater (FSW). These tissues seemed to be healthy and in good condition. Each sample was immediately frozen in liquid nitrogen and stored at –80°C until use. All animal experiments were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan).

Construction and sequencing of the EST library

Gill tissue preserved at –80°C was cut into small pieces on ice, and total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Poly(A)+ RNA was then purified from total RNA using an Oligotex-dT30 Super mRNA Purification Kit (Takara Bio Inc., Shiga, Japan), and a cDNA library was generated using a cDNA Synthesis Kit (Takara Bio Inc.) with the oligo(dT) primer 5′-$(GA)_{10}$ACTAGTCTCGAG$(T)_{18}$V-3′, according to the manufacturer's instructions. After cDNA synthesis, the cDNAs were blunted and ligated to the *Eco*RI adapter 5′-AATT-CGGCACGAGG-3′. They were digested with *Xho*I and *Eco*RI and then ligated to a pBluescript II SK(+) vector (Stratagene, La Jolla, CA, USA). *Escherichia coli* strain DH10B was transformed with the constructed cDNA library by electroporation. 5′- and 3′-terminal sequencing of the cDNA library was performed using a DYEnamic ET dye terminator kit (GE Healthcare, Little Chalfont, Bucks, UK) with the RV-M primer 5′-GAGCGGATAACA-ATTTCACACAGG-3′ and M13-47 primer 5′-CGCCAGGGTT-TTCCCAGTCACGAC-3′ on a MegaBASE4000 (GE Healthcare).

Sequence assembly and annotation

The raw sequence chromatogram files obtained were base-called using Phred software (Ewing et al., 1998). Sequences shorter than 100 bases, with a quality value of less than 25, and sequences of the vector/adapter were eliminated with Sequencher software, v4.9 (Gene Codes Co., Ann Arbor, MI, USA). All of the remaining sequences were assembled using a MIRA assembler, v3.2.1 (Chevreux et al., 2004). The assembled parameter of minimum matching between two reads was 90%, and other parameters of the MIRA assembly were default. A homology search of the assembled clusters was performed against the National Center for Biotechnology Information (NCBI) database of non-redundant proteins using BlastX. The threshold of sequence homology was defined as an E-value of $\leq 1.0\times10^{-5}$ and as homology of ≤40%. Mitochondrial and bacterial genes were removed from the assembled clusters and thus only host nuclear gene sequences were recovered. The set of read sequences used for assembly was submitted to the DDBJ (DNA Data Bank of Japan) under accession nos FY980300–FY981349, FY981351–FY981538, FY981540–FY981549 and FY981551–FY982193.

Sequencing of CA genes

Because the two CA amino acid sequences obtained from the assembled sequence were partial, the entire sequences were obtained

Table 1. Deep-sea bivalves used in the present study

Species	Location	Depth (m)	Date	Collection method
Calyptogena okutanii	Off Hatsushima seep site, Sagami Bay	800	April 2009	ROV *Hyper-Dolphin*
C. soyoae	Off Hatsushima seep site, Sagami Bay	1100	April 2009	ROV *Hyper-Dolphin*
C. phaseoliformis	Japan Trench	5800	August 2009	DSRV *Shinkai 6500*
C. nautilei	South Chamoro Seamount, Mariana Trench	1007	January 2009	ROV *Hyper-Dolphin*
Bathymodiolus septemdierum	Myojin Knoll	1303	May 2010	ROV *Hyper-Dolphin*
B. platifrons	Off Hatsushima seep site, Sagami Bay	800	April 2009	ROV *Hyper-Dolphin*
B. japonicus	Off Hatsushima seep site, Sagami Bay	800	April 2009	ROV *Hyper-Dolphin*
Mytilus sp.	JAMSTEC quay	Surface	April 2009	Human gatherer

by sequencing their corresponding EST cDNA clones. *Escherichia coli* cells transformed with CA genes were grown aerobically overnight at 37°C in Luria–Bertani medium supplemented with 100 μg ml^{-1} ampicillin. The plasmids containing the CA gene inserts were purified with a GenElute Plasmid Miniprep Kit (Sigma, St Louis, MO, USA). The DNA insert was directly sequenced by the dideoxy cycle sequencing method using a Big Dye Terminator v3.1/1.1 Cycle Sequencing Kit and an ABI PRIZM 3130 XL Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The primers used were the LF primer (5′-CAAGGCGATTAAGTTGGG-TAACGCCAGGGTTTTCCCAGTCACGAC-3′) and LR primer (5′-CTTCCGGCTCGTATGTTGTGTG-3′). The DNA sequences obtained were assembled with Sequencher software v4.9 (Gene Codes Co.).

Protein extraction and SDS-PAGE analysis

Proteins were extracted from 1 g each of the bivalve frozen tissue samples. The tissue was homogenized with 5 ml phosphate-buffered saline (PBS; 137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 10 mmol l^{-1} Na_2HPO_4 and 1.8 mmol l^{-1} KH_2PO_4) containing a protease inhibitor mix (GE Healthcare) on ice using a Potter-type homogenizer for 1 min. The pelleted blood cells (1 g wet mass) were osmotically disrupted by dilution with 5 ml of distilled water on ice. The homogenized tissues or disrupted cells were centrifuged at 600 ***g*** for 10 min at 4°C to remove cell debris. The supernatant (crude protein fraction) was transferred to a new tube and then ultracentrifuged at 100,000 ***g*** for 1 h at 4°C. The protein concentration was measured with a Qubit Protein Assay Kit (Invitrogen). All samples were diluted with the same volume of Laemmli sample buffer (Laemmli, 1970) and stored at –30°C until use. Samples were analyzed by SDS-PAGE with 12.5% polyacrylamide gel (Laemmli, 1970). After staining the gels with Coomassie Brilliant Blue R-250 (CBB), densities of the stained protein bands were determined by densitometry with ImageQuant TL software (GE Healthcare).

Identification of proteins by liquid chromatography electrospray ionization tandem mass spectrometry

Proteins were identified by liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) as previously described, with slight modifications (Ohta et al., 2004). Extracted proteins were separated on 12.5% SDS-polyacrylamide gel (Laemmli, 1970) and stained with CBB. The major stained protein bands were dissected out and washed with a washing buffer [50% (v/v) acetonitrile and 50 mmol l^{-1} ammonium bicarbonate] to remove CBB. The gel pieces were then dehydrated with 100% acetonitrile and dried in a vacuum centrifuge. They were rehydrated in 20 μl of 25 mmol l^{-1} ammonium bicarbonate containing 12.5 μg μl^{-1} trypsin (Promega, Madison, WI, USA) for 45 min on ice. After rehydration, excess solution was removed, and 20 μl of 50 mmol l^{-1} ammonium bicarbonate was added to the rehydrated gel pieces. Proteins in the gel pieces were completely digested overnight at 37°C, and the resulting peptides were transferred to a new tube and dried by vacuum centrifugation for 20 min. They were then dissolved in extraction buffer [50% (v/v) acetonitrile, 50 mmol l^{-1} ammonium bicarbonate and 0.5% (v/v) trifluoroacetic acid] by sonication for 20 min at room temperature. The peptide solution was again transferred to a clean tube. This extraction procedure was repeated three times. The peptide solution was dried by vacuum centrifugation, and the peptides were resuspended in 20 μl of 2% (v/v) acetonitrile, 98% H_2O and 1% (v/v) trifluoroacetic acid.

In on-line LC-MS/MS analysis, the peptides were separated with a MAGIC 2002 HPLC system (Michrom Bioresources, Auburn, MA, USA). They were then loaded into a nanoscale capillary column of Magic C18 reverse-phase resin (3 μm in size, 0.2×50 mm, Michrom Bioresources). They were eluted with a linear gradient of 5–85% buffer B [0.1% (v/v) formic acid and 90% acetonitrile] in buffer A [0.1% (v/v) formic acid and 2% acetonitrile] at a flow rate of 100 μl min^{-1} for 20 min. All MS/MS spectra of the eluted peptides were recorded on a LCQ Deca XP Plus ion-trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a nanospray ion source using a Fotis Tip (outside diameter 150 μm, inside diameter 20 μm) (OminiSeparo-TJ Inc., Hyogo, Japan). MS/MS spectra were acquired in the range 450–2000 *m*/*z*. The MS/MS raw data were subsequently analyzed using a TurboSEQEST program search (Ducret et al., 1998; Washburn et al., 2001). To identify the N- and C-terminal amino acid sequences of the peptide fragments, a peptide database search was performed based on the amino acid sequences of deduced proteins from the *Calyptogena* EST analysis results. The peptide sequence data obtained from LC-MS/MS were compared with those of the EST database using the TurboSEQEST program search.

Purification of CA from the gill tissue of *C. okutanii*

The CAs from *C. okutanii* were purified by affinity chromatography with a column of *p*-aminomethyl benzenesulfonamide-conjugated Sepharose HP (GE Healthcare) prepared according to the manufacturer's manual. A 1 g sample of *C. okutanii* gill tissue was homogenized in 10 ml of equilibration buffer [25 mmol l^{-1} Tris-SO_4, pH 7.5, 50 mmol l^{-1} Na_2SO_4, 100 mmol l^{-1} $NaClO_4$ with protease inhibitor mix (GE Healthcare)] on ice using a Potter-type homogenizer for 1 min. The homogenate was centrifuged at 600 ***g*** for 10 min at 4°C. The supernatant was transferred to a new tube and ultracentrifuged at 100,000 ***g*** for 1 h at 4°C. The supernatant was then applied to an affinity column pre-equilibrated with the equilibration buffer. After washing the column with 6 volumes of a washing buffer (25 mmol l^{-1} Tris-SO_4, pH 7.5, 50 mmol l^{-1} Na_2SO_4 and 200 mmol l^{-1} $NaClO_4$), CA was eluted with elution buffer (500 mmol l^{-1} $NaClO_4$ and 100 mmol l^{-1} sodium acetate, pH 5.6). Eluted fractions were neutralized with 150 mmol l^{-1} Tris-SO_4, pH 7.5, containing 300 mmol l^{-1} Na_2SO_4, and proteins in the fractions were analyzed using SDS-PAGE with 12.5% polyacrylamide gel (Laemmli, 1970). Fractions containing CA were pooled and concentrated by ultrafiltration (Microcon 10, Millipore, Billerica, MA, USA). The total amount of CA recovered was 1–3 mg.

Preparation of mouse monoclonal antibody against CA from *C. okutanii*

Mouse monoclonal antibodies were prepared following standard procedures (Galfrè and Milstein, 1981). The purified CA (300 μg) was injected into 5 week old female BALB/c mice after emulsification with Freund's complete adjuvant (Becton Dickinson, Franklin, NJ, USA). Two booster injections at 2 week intervals were performed with purified CA emulsified in Freund's incomplete adjuvant. On day 4 after the final injection, the mice were killed. The spleens were excised, and spleen cells were fused with mouse myeloma NS-1 cells using polyethylene glycol 4000 (Merck, Darmstadt, Germany). The ratio of spleen cells to NS-1 myeloma cells was 1:10. Hybridoma cells were inoculated into 96-well plates, and a mixture of hypoxanthin, aminopterin and thymidine (Sigma) was added after 24 h incubation. Following incubation for 1 week, culture fluids of hybridoma cells were screened for monoclonal antibody against *C. okutanii* CA by western blot analysis using a horseradish peroxidase-conjugated goat anti-mouse IgG as a

secondary antibody. Positive hybridoma cells were cloned using the limiting dilution method. After incubation for 1 week, culture fluids of the hybridoma cells were again assayed by western blot analysis. The cloned hybridoma cells were cultured on a large scale, and the culture fluids were stored as mouse monoclonal antibodies against *C. okutanii* CA (CokCAmab1 and CokCAmab2) at –20°C. The hybridoma cells were separated by centrifugation and stored at –80°C. All animal experiments were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan), and all protocols were approved by the institutional review board of the Animal Research Center, Yokohama City University School of Medicine.

Western blot analysis

A 1 μg sample of each protein extract of bivalve tissues was separated by 12.5% SDS-PAGE (Laemmli, 1970). The separated proteins were then transferred to a polyvinylidine difluoride membrane (Millipore) by semi-dry transfer (Nihon Eido Co., Tokyo, Japan) for 1 h at room temperature. The membrane was incubated in 4% Block Ace (Dainippon Sumitomo Pharma Co., Osaka, Japan) at room temperature for 2 h, and then in 0.4% Block Ace containing the monoclonal antibody CokCAmab1 or CokCAmab2 overnight at 4°C. The membrane was then rinsed three times in TBST [25 mmol l^{-1} Tris-HCl pH 7.4, 137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl and 0.05% (v/v) Tween-20] and incubated for 1 h at room temperature with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Biosource, Camarillo, CA, USA) for CokCAmab1 or a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Biosource) for CokCAmab2. The membrane was washed three times in TBST, and the bound antibody was detected by incubation with 0.2 mg ml^{-1} diaminobenzidine in 50 mmol l^{-1} Tris-HCl buffer (pH 8.0) containing 0.1% H_2O_2 or NBT/BCIP solution (Hoffmann-La Roche, Basel, Switzerland).

Measurement of CA activity

The gill tissues were homogenized with 5–20 volumes of 25 mmol l^{-1} veronal buffer [25 mmol l^{-1} barbital buffer (Wako Pure Chemical Industries, Osaka, Japan), 5 mmol l^{-1} EDTA, 5 mmol l^{-1} dithiothreitol (DTT) and 10 mmol l^{-1} $MgSO_4$, pH 8.2] using a Potter-type homogenizer. The homogenate was centrifuged at 600 ***g*** for 10 min at 4°C. The supernatant was transferred to a new tube and ultracentrifuged at 100,000 ***g*** for 1 h at 4°C to remove insoluble proteins. The protein concentration of the soluble fraction was measured with a Qubit Protein Assay Kit (Invitrogen). CA activity was measured according to the method of Weis et al. (Weis et al., 1989). The soluble protein fraction or the CA solution containing two co-purified CAs was diluted 100-fold with 25 mmol l^{-1} veronal buffer. A 3 ml sample of the assay mixture consisting of 1 ml of 50 mmol l^{-1} veronal buffer, 1 ml of CO_2-saturated distilled water and 0.99 ml of 25 mmol l^{-1} veronal buffer in a plastic cell was preincubated at 4°C and stirred constantly with a small magnetic stirring bar. The reaction was initiated by adding 10 μl of the diluted supernatant. The rate of change in pH was followed from pH 8.00 to 7.50 with a pH meter connected to a personal computer equipped for digital data acquisition and analysis. Each sample was assayed in triplicate, and the results were averaged. A unit of CA activity was defined according to the equation $(\Delta pH_s - \Delta pH_b)$ min^{-1} mg^{-1} protein, where ΔpH_s is the rate of pH change in the protein sample, and ΔpH_b is the rate of pH change in an identical sample boiled for 5 min to eliminate enzyme activity (Weis et al., 1989). This unit is hereafter abbreviated as ΔpH min^{-1} mg^{-1} protein. Acetazolamide (Sigma) was used as a specific inhibitor of CA. For inhibition assays, the purified CA was dissolved in veronal buffer to adjust it to the desired concentration (i.e. 1, 0.1 mmol l^{-1}, etc.) (Kochevar and Childress, 1996), and 1 ml of the resulting solution was substituted for 1 ml of the 50 mmol l^{-1} veronal buffer in the above protocol. The inhibition was evaluated by calculating the percentage of activity compared with CA activity without the inhibitor.

Alignment of the CA amino acid sequences of *C. okutanii*

Two human CA sequences, human CAI and CAII, with respective accession numbers P00915 and P00918, were retrieved from the NCBI server (http://www.ncbi.nlm.nih.gov/). The sequences of human CAs and *C. okutanii* CAs were aligned with CLUSTALW (Thompson et al., 1994) using default parameters, followed by manual editing of the resulting alignments to ensure that the optimal alignment was obtained.

Table 2. Genes highly expressed in gill tissue

Local ID	EST	Gene homologue	Source organism	NCBI accession no.	*E*-value
cokg051	69	Carbonic anhydrase 1	*Monodelphis domestica*	NP_001028142.1	5.00E–53
cokg057	23	Carbonic anhydrase 2	*Mus musculus*	NP_033931.4	7.00E–59
cokg040	20	Carbonic anhydrase 1	*Monodelphis domestica*	NP_001028142.1	2.00E–57
cokg060	17	Putative ribosomal protein S15	*Barentsia elongata*	ABW90420.1	5.00E–51
cokg061	15	Hemoglobin III	*Calyptogena nautilei*	BAD34605.1	6.00E–21
cokg050	11	Carbonic anhydrase 2	*Mus musculus*	NP_033931.4	2.00E–45
cokg041	9	Hemoglobin chain I	*Calyptogena kaikoi*	BAD34601.1	3.00E–20
cokg043	7	Ferritin	*Sinonovacula constricta*	ACZ65230.1	2.00E–74
cokg021	7	Hemoglobin II	*Calyptogena soyoae*	BAD34604.1	3.00E–72
cokg042	6	Predicted thymosin beta-12-like	*Amphimedon queenslandica*	XP_003385840.1	3.00E–12
cokg045	6	Hemoglobin I	*Calyptogena soyoae*	BAD34603.1	4.00E–76
cokg044	5	Heat shock protein 90	*Crassostrea gigas*	ABS18268.1	1.00E–45
cokg024	5	Histone H3.2	*Culex quinquefasciatus*	XP_001870860.1	1.00E–68
cokg046	4	Ribosomal protein S7	*Argopecten irradians*	AAN05602.1	8.00E–77
cokg020	4	Receptor for activated C-kinase	*Pinctada fucata*	ACJ06767.1	1.00E–90
cokg022	4	40S ribosomal protein S25	*Branchiostoma belcheri*	Q8ISN9.1	1.00E–28
cokg023	4	Ribosomal protein L44	*Chlamys farreri*	AAM94276.1	3.00E–38
cokg027	4	Ribosomal protein rps21	*Eurythoe complanata*	ABW23211.1	8.00E–35
cokg028	4	qm-like protein	*Crassostrea ariakensis*	ACO07302.1	1.00E–100
cokg029	4	Putative ribosomal protein L29	*Barentsia elongata*	ABW90402.1	1.00E–16

Expression sequence tag (EST) values indicate the number of clones.

Immunofluorescence microscopy

For immunofluorescence microscopy, small pieces (5×5×5 mm) of gill tissues of *C. okutanii* were fixed with 4% paraformaldehyde (PFA) in FSW overnight at 4°C. Subsequently, they were washed in PBS containing 15% sucrose for 6 h at 4°C and then in PBS containing 30% sucrose overnight at 4°C. The tissue samples were then embedded in OCT compound (Sakura Finetek Japan Co., Tokyo, Japan) and frozen in hexane cooled with liquid nitrogen. Frozen sections (4 μm) were placed on MAS-coated glass slides (Matsunami, Osaka, Japan). The sections were incubated with 4% Block Ace for 1 h at room temperature in a moist chamber. Subsequently, the sections were incubated overnight at 4°C in 0.4% Block Ace containing the CokCAmab1 (diluted 1:10) and anti-GroEL rabbit antibody (diluted 1:1000, Sigma) to detect symbionts. The sections were then washed three times in PBS and incubated again with the secondary antibodies (1:1000 diluted Alexa Fluor 488-conjugated anti-mouse goat IgG and 1:1000 diluted Alexa Fluor 647-conjugated anti-rabbit goat IgG, Invitrogen) in 0.4% Block Ace for 1 h at room temperature in a dark moist chamber. After incubation, the sections were rinsed three times in PBS. DNA was stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Dojindo, Kumamoto, Japan). The sections were mounted on a glass slide with Fluoromount (Diagnostic BioSystems, Pleasanton, CA, USA), covered with a coverslip and observed under a confocal laser scanning microscope system (Fluoview FV500 system, Olympus, Tokyo, Japan). Micrographs were processed with Adobe Photoshop CS5.1 (Adobe Systems Inc., San Jose, CA, USA).

Immunoelectron microscopy

Immunoelectron microscopy was performed as detailed elsewhere (Yamashita et al., 2009). Gill tissues (5×5×5 mm) of *C. okutanii* were fixed with 4% PFA in FSW for 30 min at 4°C and then cut into small pieces (2×2×2 mm). They were additionally fixed with 4% PFA in artificial seawater (ASW) with pH adjusted to 8.5 with sodium hydroxide, for 2 months at 4°C. Samples were then washed with ASW, pH 8.5. The specimens were dehydrated in a graded series of *N*,*N*-dimethylformamide (Wako Pure Chemicals) and embedded in LR-White (London Resin Company, Reading, Berks, UK). Ultrathin sections were prepared using a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria) with a diamond knife.

Ultrathin sections were mounted on a nickel grid pretreated with Neoprene W (Nisshin EM, Tokyo, Japan), dried and stored at room temperature until use. The sections were incubated with 20 mmol l^{-1} Tris-HCl, pH 9.0, in an Eppendorff tube (1.5 ml) preheated at 95°C on a block heater for 4 h. After cooling to room temperature, the sections were washed with TBS (25 mmol l^{-1} Tris-HCl pH 7.4, 137 mmol l^{-1} NaCl and 2.7 mmol l^{-1} KCl) and treated with a blocking solution (1% BSA in TBS) for 30 min at room temperature. The sections were then incubated with CokCAmab1 diluted 1:50 with blocking solution overnight at 4°C. The sections were washed with TBS and incubated with 20 nm colloidal gold-labeled goat anti-mouse IgG antibodies for 1 h. Before this treatment, the gold-labeled antibodies were diluted 1:10 with blocking solution for 1 h at room temperature. After washing with TBS and then with distilled water, the sections were treated with 2% glutaraldehyde containing 0.05% tannic acid in 0.1 mol l^{-1} phosphate buffer (PB), pH 5.5, for 5 min and washed with distilled water. The sections were post-fixed with 1% OsO_4/0.1 mol l^{-1} PB (pH 7.4) for 5 min, washed with distilled water and stained with 2% uranyl acetate aqueous solution for 5 min and lead stain solution (Sigma) for 1 min. Ultrathin sections were then observed in a transmission electron microscope (JEM-1210, JEOL, Akishima, Japan) at an acceleration voltage of 100 kV.

RESULTS

EST analysis of gill tissue from *C. okutanii*

A total of 1152 cDNA clones of gill tissue of *C. okutanii* were randomly selected and both the 5′- and 3′-terminal ends of the cDNA clones were sequenced. A total of 2297 base-called sequences were obtained (see supplementary material Table S1). After discarding low-quality and short (<100 bp) sequences and trimming off vector sequences, 1891 sequences with an average length of 453 bp were obtained. Subsequently, these sequences were assembled into 222 clusters, comprising 161 contigs and 61 singletons. Of the 222 clusters, 30% (67; 60 contigs and 7 singlets) showed significant similarity ($E \leq 1.0\times10^{-5}$) to protein-encoding genes in the NCBI non-redundant database, but 70% of them (155; 101 contigs and 54 singlets) showed no similarity to any reported genes. The most and second-most abundant clusters were similar to CA genes, although they were distinct from each other (Table 2). They were followed in abundance by ribosomal protein S15 and hemoglobin III (Table 2).

CA protein in gill tissue of *C. okutanii*

Total proteins extracted from the gill, blood cells, mantle and foot of *C. okutanii* were analyzed by SDS-PAGE (Fig. 1A). Although electrophoretograms of proteins from the mantle and foot were similar, those from the gill and blood cells differed from them and

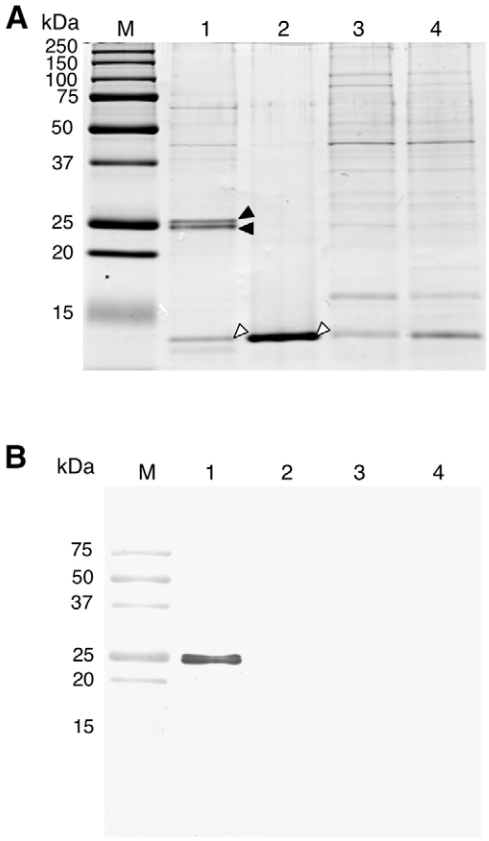

Fig. 1. Protein electrophoretogram and carbonic anhydrase (CA) in tissues of *Calyptogena okutanii*. (A) Protein from various tissue extracts analyzed by SDS-PAGE. Tissue extracts (2 μg protein per lane) were separated by 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (CBB). Three and one major bands were detected in the gill tissue (lane 1) and blood cells (lane 2), respectively. Black arrowheads, CA; white arrowheads, hemoglobin. (B) CA detected by western blot analysis. Two CA bands for CokCAg1 and CokCAg2 were detected in the gill tissue with anti-CA mouse monoclonal antibody (CokCAmab1). M, molecular marker; 1, gill tissue; 2, blood cells; 3, mantle tissue; 4, foot tissue.

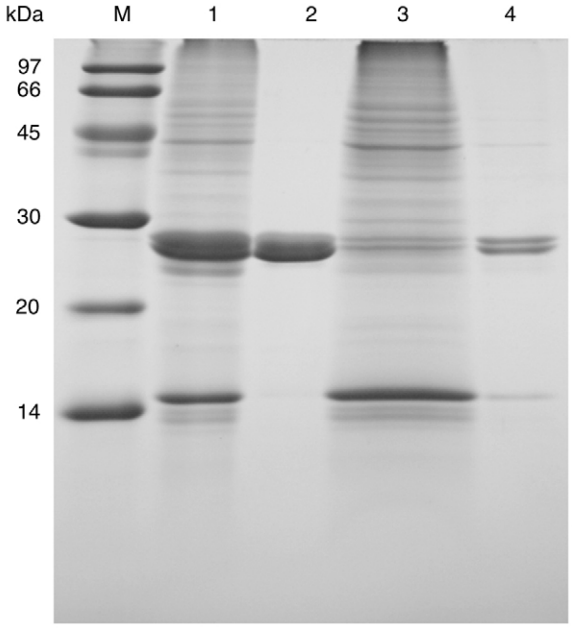

Fig. 2. Purification of *C. okutanii* CA by affinity chromatography. Protein fractions (5 µg each) from several steps of CA purification were separated by 15% SDS-PAGE and stained with CBB. M, molecular marker; 1, gill crude extract; 2, purified CAs (eluate); 3, fraction passed through the column; 4, wash fraction.

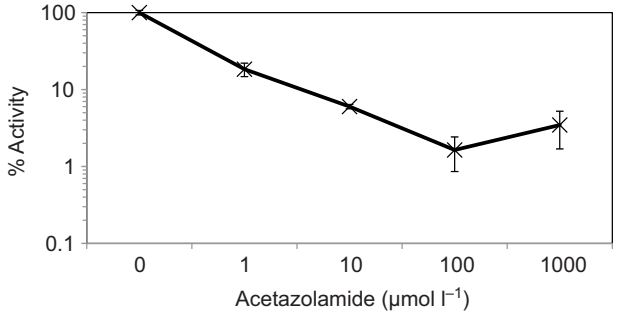

Fig. 3. Inhibition of CA activity by acetazolamide. Activity (mean ± s.d.) of the affinity-purified CA of *C. okutanii* was assayed in buffer containing various concentrations of the CA inhibitor acetazolamide. CA activity was inhibited by acetazolamide in a concentration-dependent manner.

from each other. Three major bands were found in electrophoretograms of the gill extract. Two bands were ~25 kDa (Fig. 1A, black arrowheads), and another was ~13 kDa (Fig. 1A, white arrowheads in gill extract). The band of ~13 kDa was the most prominent in blood cells (Fig. 1A, white arrowheads). The amino acid sequences of the two bands of ~25 kDa in the gill were determined to be those of CAs, and the ~13 kDa bands in blood cells and gill were hemoglobin based on LC-MS/MS results with the protein database from EST analysis. Few other peptides apart from CAs were detected in the two ~25 kDa bands, and the abundance of these peptides was low, according to LC-MS/MS analysis (data not shown). The upper and lower bands of the CAs were, respectively, designated as CokCAg1 and CokCAg2. Because the two CA amino acid sequences were only partial, the entire sequences were obtained by sequencing their corresponding EST cDNA clones (accession nos: CokCAg1, AB731736; CokCAg2, AB731737). The amino acid sequence of CokCAg1 was 255 amino acid residues with an estimated molecular mass of 26,919 Da, and that of CokCAg2 was 254 amino acid residues with a molecular mass of 26,471 Da. The homology between the amino acid sequences of CokCAg1 and CokCAg2 was 85.8%. Although the *Calyptogena* symbiont also has one CA gene (Kuwahara et al., 2007; Newton et al., 2007), the amino acid sequence of the symbiont CA is different from those of CokCAg1 and CokCAg2.

Purification and activity measurement of *C. okutanii* CA

To examine the CA activity of CokCAg1 and CokCAg2, they were purified from the gill tissue of *C. okutanii* by affinity chromatography. These CAs were purified as soluble proteins. Although most other proteins were removed, these two CAs were co-purified (Fig. 2) and could not be separated. The activity of the co-purified CA mixture was 100.8±6.8 ΔpH min^{-1} mg^{-1} protein. CA activity was inhibited by the specific inhibitor acetazolamide in a dose-dependent manner (Fig. 3). The inhibition reached a plateau at a concentration of 100 µmol l^{-1} acetazolamide (Fig. 3). Although the co-purified CA activity was detected, it was unclear whether the two CAs had different activities. To compare the active site, their amino acid sequences were aligned with the human cytosolic CA isozymes CA I and CA II (Fig. 4). CAs of *C. okutanii* were shown to have three conserved His residues (corresponding to His94, His96 and His119 of human CAII) for binding zinc (labeled 'Z' in Fig. 4). CokCAg1 and CokCAg2 showed three amino acid substitutions in the active site, at Asn62Thr, His64Thr and Asn67Lys (boxes in Fig. 4).

Monoclonal antibodies against CA of *C. okutanii*

The co-purified CA mixture was used for raising mouse monoclonal antibodies. Western blot analysis showed that CokCAmab1 was specific to the CA in the gill tissue and did not react with other proteins (Fig. 1B). CokCAmab1 and CokCAmab2 reacted with both CokCAg1 and CokCAg2 (Fig. 1B; see supplementary material Fig. S1). However, no monoclonal antibody distinguishing the two CAs was obtained.

CA in gill tissues of chemosynthetic bivalves

To examine whether CA was specifically expressed in the gill tissue of bivalves that harbor chemosynthetic bacteria, we analyzed CA protein in the gill tissues of bivalves containing thioautotrophic symbionts (*C. okutanii*, *C. soyoae*, *C. phaseoliformis*, *C. nautilei* and *Bathymodiolus septemdierum*) in comparison with CA protein of bivalves containing methanotrophic symbionts (*B. japonicus* and *B. platifrons*) and that of a non-symbiotic bivalve (*Mytilus* sp.).

Soluble and insoluble protein fractions of gill tissues of the bivalves were analyzed using SDS-PAGE (Fig. 5A). Protein band patterns of the *Calyptogena* species were similar. Two intensely stained bands of ~25 kDa, which reacted with the CokCAmab1 of *C. okutanii*, were similarly found in the soluble fraction of *C. soyoae* (Fig. 5A, lane 3, black arrowheads). In contrast, only one corresponding band was found in *C. phaseoliformis* and *C. nautilei* (Fig. 5A, lane 5 and lane 7). While *B. septemdierum* had one intensely CBB-stained band with a molecular mass of ~28.5 kDa, both *B. platifrons* and *B. japonicus* had two CBB-stained bands with a slightly higher molecular mass (~29 kDa; Fig. 5A, lane 9, lane 11 and lane 13). However, no such intensely CBB-stained band was found in *Mytilus* sp. (Fig. 5A, lanes 15 and 16).

In western blot analysis with CokCAmab1, positive bands were detected only in protein extract from *C. okutanii*, *C. soyoae*, *C. phaseoliformis* and *C. nautilei*, but not in *Bathymodiolus* mussels

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                    10          20          30          40          50          60
CokCAg1       -MS--WGYTQ ANGPSTWAAS FPGAGGAAQS PIDIKTNKLK HDGSLASIAV NYKSEAQYTA
CokCAg2       -MS--WGYTQ ANGPSTWAAS FPGAGGSAQS PVDIQTNAVK HDGGLASMAV NYKAEAQYTA
Human CAI     MASPDWGYDD KNGPEQWSKL YPIANGNNQS PVDIKTSETK HDTSLKPISV SYNPATAKEI
Human CAII    -MSHHWGYGK HNGPEHWHKD FPIAKGERQS PVDIDTHTAK YDPSLKPLSV SYDQATSLRI

                    70          80          90          100         110         120
                                                  Z Z                       Z
CokCAg1       TNTGTGFKVD LQK---ASEL SGGPLNASYK LEQFHWHWGS SDSTGSEHTI DGKQYAAELH
CokCAg2       TNTGTGFKVD LQN---VSEL SGGPLNASYK LVQFHWHWGP SDSAGSEHTI NGKQYAAELH
Human CAI     INVGHSFHVN FEDNDNRSVL KGGPFSDSYR LFQFHFHWGS TNEHGSEHTV DGVKYSAELH
Human CAII    LNNGHAFNVE FDDSQDKAVL KGGPLDGTYR LIQFHFHWGS LDGQGSEHTV DKKKYAAELH

                    130         140         150         160         170         180
CokCAg1       LVHSN-SKYS SFSEASSKPD GLAVIAVMIE AGSANEAFQD MVAVSKNVNG CGDTCKVKKS
CokCAg2       LVHSN-SKYS SFSEAVSQPD GLAVIAVMIE AGSANQAFQE MVDASKKVNA CGATCSVKNS
Human CAI     VAHWNSAKYS SLAEAASKAD GLAVIGVLMK VGEANPKLQK VLDALQAIKT KGKRAPFTN-
Human CAII    LVHWN-TKYG DFGKAVQQPD GLAVLGIFLK VGSAKPGLQK VVDVLDSIKT KGKSADFTN-

                    190         200         210         220         230         240
CokCAg1       FNPSVLLPAR SNEFYTYHGS LTTPPCNESV QWIVLRQSVQ YSSGQLGALR SLASCEAGG-
CokCAg2       FNPSVLLPGS TSEFYTYHGS LTTPPCNECV QWIVLSQSVQ YSSGQLSALR SLASCEAGG-
Human CAI     FDPSTLLP-S SLDFWTYPGS LTHPPLYESV TWIICKESIS VSSEQLAQFR SLLSNVEGDN
Human CAII    FDPRGLLP-E SLDYWTYPGS LTTPPLLECV TWIVLKEPIS VSSEQVLKFR KLNFNGEGEP

                    250         260
CokCAg1       --CIITNHRP VMPLGGRAVS ASFK
CokCAg2       --CITTNCRP VMPLGGRPVA ASF-
Human CAI     AVPMQHNNRP TQPLKGRTVR ASF-
Human CAII    EELMVDNWRP AQPLKNRQIK ASFK
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Fig. 4. Alignment of CA amino acid sequences of *C. okutanii* with human CA I and II. The amino acid sequences of two CAs of *C. okutanii* were compared with human CA I (accession no. P00915) and CA II (accession no. P00918). Black arrowheads, the active site of amino acid residues involved in hydrogen network formation with zinc-bound water including His64. Z, three conserved His residues for binding zinc. Boxes indicate amino acid residues of *C. okutanii* CAs that differed from those of human CA II in the active site. White arrowheads, amino acid residues of the hydrophobic pocket; black circles, hydrogen binding site of acetazolamide; gray-shaded residues, conserved identical residues.

and *Mytilus* (Fig. 5B). However, the protein bands in *Bathymodiolus* mussels were positively stained with CokCAmab2 (see supplementary material Fig. S1) in western blot analysis. This result indicates that these intensely CBB-stained bands in *Bathymodiolus* species were CAs. Densitometric analysis of the SDS-PAGE protein profile showed that the percentage of CA in total soluble proteins in bivalves harboring thioautotrophic symbionts (*Calyptogena* clams and *B. septemdierum*) was high (43–72%, Table 3).

In *C. okutanii* and *C. soyoae*, the two bands corresponding to CokCAg1 and CokCAg2 were significantly less dense in the

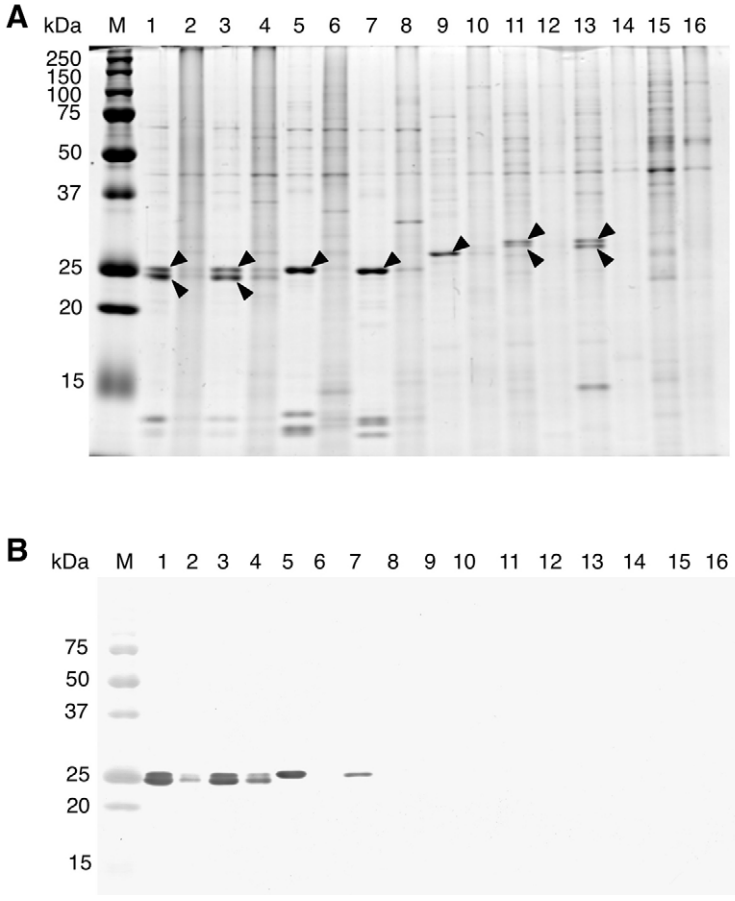

Fig. 5. SDS-PAGE and western blot analysis of CAs in gill tissues of deep-sea and shallow-sea bivalves. Each lane was loaded with 1 µg of gill tissue protein, and the gel was stained with CBB in SDS-PAGE analysis (A). For western blot analysis, the monoclonal mouse antibody CokCAmab1 of the gill tissue extracts of various deep-sea and shallow-sea bivalves was used (B). Lanes 1–10, bivalves harboring thioautotrophic symbionts. Lanes 11–14, bivalves harboring methanotrophic symbionts. Lanes 15 and 16, *Mytilus* sp. Lanes 1 and 2, *C. okutanii*; lanes 3 and 4, *C. soyoae*; lanes 5 and 6, *C. phaseoliformis*; lanes 7 and 8, *C. nautilei*; lanes 9 and 10, *Bathymodiolus septemdierum*; lanes 11 and 12, *B. platifrons*; lanes 13 and 14, *B. japonicus*. Odd numbers, soluble fraction; even numbers, insoluble fraction.

Table 3. Carbonic anhydrase activity of bivalve gill soluble protein and specific activity

Type of symbiont	Species	CA activity (ΔpH min^{-1} mg^{-1} total protein)	CA protein abundance (%)
Thioautotrophs	*Calyptogena okutanii*	19.5±3.8 (2)	59.4
	C. soyoae	17.3±7.8 (2)	72.1
	C. phaseoliformis	3.3±0.9 (2)	43.0
	C. nautilei	21.0±3.7 (1)	51.4
	Bathymodiolus septemdierum	4.8±3.7 (3)	51.5
Methanotrophs	*B. platifrons*	2.8±3.4 (2)	26.6
	B. japonicus	5.6±3.0 (2)	36.0
Non-symbiotic	*Mytilus* sp.	ND (3)	ND

Carbonic anhydrase (CA) activity was measured in a soluble protein fraction from each gill tissue homogenate.
CA activity values are means ± s.d. of three assays in *N* individuals (in parentheses), and the results were averaged.
CA protein abundance was calculated from the CBB-stained density of CA bands as a percentage of the total CBB-stained band intensity of the soluble fraction on the SDS-PAGE gel of Fig. 5A.
ND, not detected.

insoluble protein fraction (Fig. 5A) and were thought to be a contamination of soluble CA. In *C. phaseoliformis* and *C. nautilei*, a CokCAmab1-positive CA band, which corresponded to the intensely CBB-stained band in SDS-PAGE, was detected only in the soluble protein fraction (Fig. 5).

CA activity in gill tissue of chemosynthetic bivalves

CA activity was detected in the soluble fraction of all gill tissue homogenates from symbiotic clams and mussels, ranging from 2.8 to 21.0 ΔpH min^{-1} mg^{-1} protein in *B. platifrons* and *C. nautilei*, respectively (Table 3). CA activity was not detected in the insoluble membrane protein fraction of gill tissues of *C. okutanii* and *C. soyoae* (data not shown). CA activity was significantly higher in *Calyptogena* clams, except for *C. phaseoliformis*, than in *Bathymodiolus* mussels (P<0.05, t-test). The activity in *C. phaseoliformis* (3.3±0.9 ΔpH min^{-1} mg^{-1} protein) was markedly lower than that in other *Calyptogena* clams (Table 3). Three *Bathymodiolus* mussels, which harbor thioautotrophic or methanotrophic symbionts, exhibited lower activities, ranging from 2.8 to 5.6 ΔpH min^{-1} mg^{-1} protein. No CA activity was detected in the non-symbiotic mussel *Mytilus* sp. (Table 3).

Localization of CA in *C. okutanii* gill tissue

To examine the localization of CA in the gill tissue of *C. okutanii*, frozen sections of gill tissue were immunostained with CokCAmab1 and observed by confocal laser scanning microscopy. In gill tissue sections from *C. okutanii*, the symbionts were detected with the rabbit polyclonal anti-bacterial GroEL antibody (red fluorescence in Fig. 6B,D). Symbionts were specifically localized in the bacteriocytes of epithelial cells inside the internal symbiont zone of gill tissue (Fig. 6A,B). CA was detected only in bacteriocytes (Fig. 6B,D). No CA was detected in the external asymbiotic zone (Fig. 6A,B) and in the intermediate cells by immunoelectron microscopy (data not shown). In bacteriocytes, immunoelectron microscopy showed that CA was detected neither on cell membranes nor on symbiosome membranes but exclusively in the cytoplasm

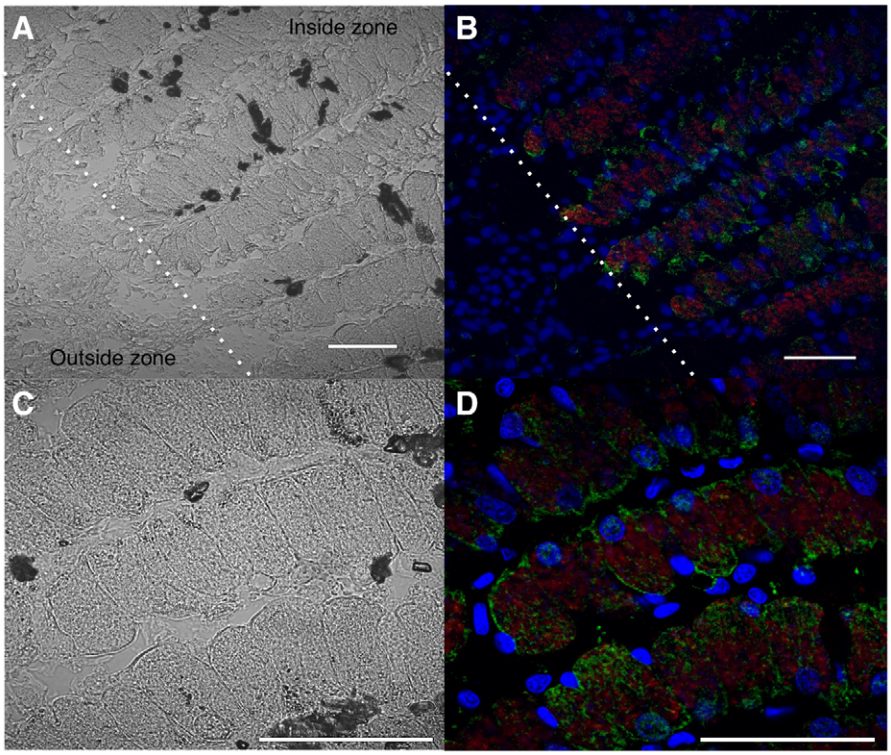

Fig. 6. Localization of CA in gill epithelial tissue. Confocal laser scanning micrographs of transverse sections of gill filaments. (A,C) Differential interference contrast images; (B,D) immunofluorescence images. CA was detected with CokCAmab1 as green fluorescence. The symbionts were detected with anti-GroEL rabbit antibody as red fluorescence. The right side of the dotted line is the inner region of the gill tissue containing bacteriocytes, and the left side is the outer region lacking bacteriocytes. The host cell nuclei and symbiotic bacterial nucleoids were stained with DAPI as blue fluorescence. Scale bars, 50 μm. CokCAg1 and CokCAg2 were detected exclusively in the cytoplasm of bacteriocytes.

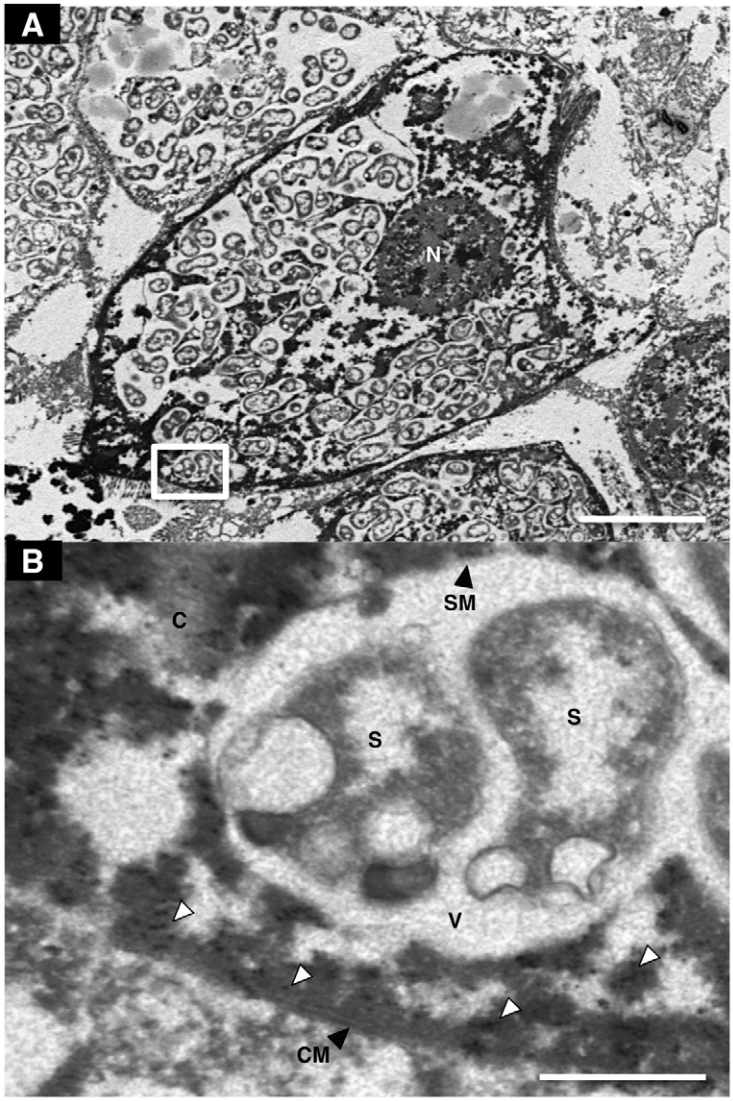

Fig. 7. Immunoelectron microscopy of CA in a gill epithelial cell of *C. okutanii*. (A) Immunoelectron micrographs of a gill epithelial cell. (B) High-magnification electron micrograph of the region indicated by the white square in A. CA was detected as 20 nm gold particles (white arrowheads in B). N, nucleus; S, symbiont; V, vacuole; C, cytoplasm; CM, cell membrane; SM, symbiosome membrane. Scale bars, 5 µm in A and 500 nm in B.

(Fig. 7B). CokCAmab1 did not react with the symbiont cells in the symbiosome (Fig. 7B).

DISCUSSION

Specific and abundant expression of functional CA in gill tissue of *C. okutanii*

In *C. okutanii*, two CA proteins were exclusively localized and abundantly expressed in the bacteriocytes of gill tissue as soluble proteins (Figs 1, 5, 6), and CA activity was also detected in the co-purified and soluble protein fraction (Table 3). However, it was not clear whether the two CAs of *C. okutanii* were different in their enzymatic activity, because they could not be separated (Fig. 2). To clarify this question, the active site of the two CAs in *C. okutanii* were compared with human cytosolic CA isozymes CA I and II, the functions and three-dimensional structures of which have been characterized (Fig. 4) (Kannan et al., 1975; Liljas et al., 1972; Nair et al., 1991). In the active site of human CA II, Tyr7, Asn62, His64, Asn67, Thr199 and Thr200 form a network of hydrogen bonds to zinc (corresponding residues are shown as black arrowheads at positions 8, 63, 65, 68, 202 and 203 in Fig. 4) (Fisher et al., 2007). However, three amino acids of both CokCAg1 and CokCAg2 were substituted in the network, at Asn62Thr, His64Thr and Asn67Lys (boxes in Fig. 4). Histidine 64 is essential for human CA to function as an efficient proton shuttle (Fisher et al., 2007). Although the amino acid substitution of His64Ala in human CA II was shown to reduce its proton transfer activity (Tu et al., 1989), significant CA activity was detected for the purified CokCAg1 and CokCAg2 mixture (Table 3, Fig. 3). This indicates that both CAs should be active. The ancestral *Calyptogena* clam probably had a single CA gene. After the divergence of the *C. okutanii* and *C. soyoae* lineage and the *C. phaseoliformis* and *C. nautilei* lineage, the CA gene was duplicated in the former. Both of the duplicates remain active in the extant clams in the lineage. It is not clear whether the duplication was required for the functional divergence or it was more advantageous for producing larger amounts of the enzyme. The effect of the substitutions of these important amino acid residues on CA activity is interesting but remains to be elucidated in future studies.

Expression of CA in the gill tissues of *Calyptogena* clams and *Bathymodiolus* mussels

In gill tissues, abundant CAs were detected in *Calyptogena* clams and *Bathymodiolus* mussels with monoclonal antibodies (Fig. 5B; see supplementary material Fig. S1). The CA content of the total gill soluble protein of chemosynthetic bivalves was 26–72%

(Fig. 5A, Table 3). Neither a band corresponding to CA on SDS-PAGE nor CA activity was detected in the non-symbiotic mussel *Mytilus* sp. (Fig. 5, Table 3; see supplementary material Fig. S1). In C3 plant leaves, CA functions in CO_2 uptake and is abundant, comprising 2% of the total soluble leaf protein (Coleman, 2000; Okabe et al., 1984). The CA content in the chemosynthetic bivalves examined here was much higher than that in plant leaves. CA in photosymbiotic cnidarians harboring symbiotic algae has been reported to range from 0.106 to 7.517 ΔpH min^{-1} mg^{-1} protein (Weis et al., 1989), which is lower than the rate in bivalves harboring thioautotrophic symbionts (Table 3). In siboglinid tubeworms, CA activity in the troposome, which is a unique organ harboring chemoautotrophic symbionts, ranges from 1.22±0.69 to 7.11±4.63 ΔpH min^{-1} mg^{-1} protein (Kochevar and Childress, 1996). The higher activity of CA in the gills of *Calyptogena* clams is in agreement with the hypothesis that CA functions in C_i uptake or supply for symbionts.

It is interesting that CA activity in the gill tissue of *B. japonicus* and *B. platifrons* was comparable to that in *B. septemdierum* and was significantly higher than that in the non-symbiotic mussel *Mytilus* sp. (Table 3) *Bathymodiolus japonicus* and *B. platifrons* harbor methanotrophic γ-proteobacteria symbionts, which utilize only methane as a carbon source. The symbionts oxidize methane to generate energy, produce various organic compounds and finally produce CO_2 (Kochevar et al., 1992). The free-living methanotrophic bacterium *Methylomonas methanica*, a member of the γ-proteobacteria, oxidizes 55% of incorporated methane to produce organic compounds, and 45% of the incorporated methane is converted to CO_2 (Templeton et al., 2006). It is possible that the CA of bivalves harboring methanotrophic symbionts is a function in the elimination of CO_2 produced by the symbionts.

C_i uptake from seawater

Bivalves harboring thioautotrophic symbionts eliminate respiratory CO_2. Respiratory CO_2 may be recycled in thioautotrophic bivalves, but the recycled CO_2 is not enough to support host growth. Thioautotrophic bivalves must take up C_i from seawater to supply it to the symbionts. However, the mechanism of C_i uptake by chemosynthetic bivalves is still not completely understood. The deep-sea vestimentiferan tubeworm *Riftia pachyptila*, which harbors thioautotrophic symbionts in a special organ called the trophosome, is known to take up C_i from seawater (Goffredi et al., 1997). It expresses high levels of cytosolic CA in a respiratory organ called the branchial plume and in the trophosome (De Cian et al., 2003; Sanchez et al., 2007). In general, C_i in the seawater exists mostly as HCO_3^-, which does not penetrate cell membranes. It was reported that the partial pressure of CO_2 (P_{CO_2}) in the environment surrounding *Riftia pachyptila* is high (P_{CO_2}=2.89±0.64 kPa), and the total CO_2 (ΣCO_2) in seawater among *R. pachyptila* is also high (4.70±0.54 mmol l^{-1}) (Childress et al., 1993b). The pH of the surrounding seawater was found to be 6.22±0.14 (Childress et al., 1993b). *Riftia pachyptila* acquires CO_2 from the environment, and acquired CO_2 is transmitted to the branchial plume cells *via* diffusion (Goffredi et al., 1997). In branchial plume cells, CO_2 is immediately converted to HCO_3^- to reach the C_i equilibrium at intraplume pH (Goffredi et al., 1997). This reduces the CO_2 concentration in the plume cytoplasm and enhances the diffusion of surrounding CO_2 into plume cells (Goffredi et al., 1997). If *Calyptogena* clams obtain CO_2 by diffusion through the gill tissue like *R. pachyptila*, the P_{CO_2} in the seawater at the site of a *Calyptogena* clam colony should also be high. However, the ΣCO_2 and pH values of sea-floor water at the *C. okutanii* colony site off Hatsushima Island, Sagami Bay, Japan, were reported to be about 2.3 mmol l^{-1} and pH 7.7, respectively, both of which are common in deep-sea environments (pH value; unpublished data of T. Fukuba, JAMSTEC) (Tsunogai et al., 1996). Using these data, P_{CO_2} at the *Calyptogena* colony site was estimated to be 0.1 kPa with the seawater carbon calculator CO2Calc (Lewis and Wallace, 1998). These environmental data suggest that *C. okutanii* is unlikely to take up CO_2 by diffusion. Therefore, another mechanism of C_i uptake likely occurs in the gill tissue of *C. okutanii*, and the cytosolic CAs may participate in C_i uptake and transport to the symbionts.

Role of cytosolic CAs in C_i uptake and transport processes in the gill tissue of *C. okutanii*

In general, the role of gills in marine organisms is to release C_i produced during respiration into the environmental seawater (Eckert and Randall, 1978). However, in symbiotic bivalves harboring thioautotrophic bacteria, C_i is required for autotrophic carbon fixation by symbionts in gill epithelial cells (Childress et al., 1991). CokCAg1 and CokCAg2 were shown to be localized exclusively in the bacteriocytes and not detected in asymbiotic cells (Fig. 6A,B). In cnidarian–zooxanthellae symbiosis, it has been reported that cytosolic CA is localized on or near the vacuolar membrane surrounding the zooxanthellae (Weis, 1993). Unlike cnidarian symbiosis, CokCAg1 and CokCAg2 were found in the cytoplasm but not on the cell surface or symbiosome membrane of bacteriocytes, although we expected CA to localize on the cell membrane (Fig. 6 and Fig. 7B). Furthermore, CA activity was not detected in the insoluble membrane protein fraction of the gill tissue of *C. okutanii*. If CA plays a role in C_i uptake from seawater to the cytoplasm, it should be localized on the cell surface. Therefore, CokCAg1 and CokCAg2 do not seem to function directly in C_i uptake from seawater. To explain the C_i uptake from seawater by bacteriocytes, we assume that there are two processes of C_i uptake, involving bicarbonate transporter and membrane-associated CA (Fig. 8). A bicarbonate transporter (electrogenic Na/HCO_3^-

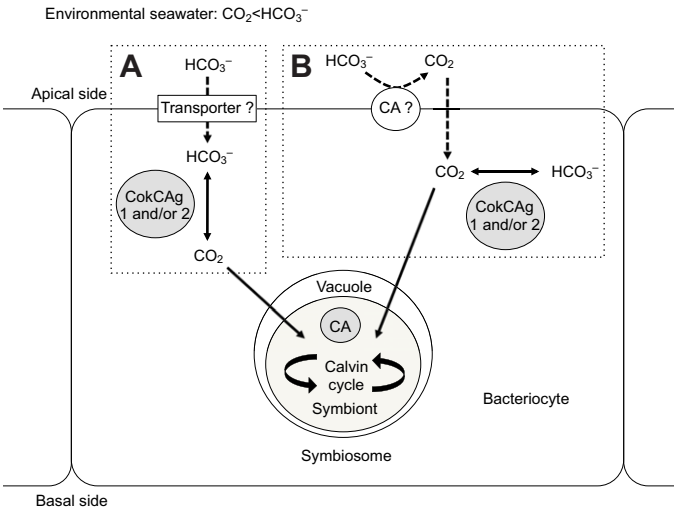

Fig. 8. Possible role of CA in bacteriocytes in the gill of *C. okutanii*. CokCAg1 and/or CokCAg2 catalyze the conversion between HCO_3^- and CO_2 in the cytoplasm. CO_2 is transferred from the cytoplasm to the symbiont in the symbiosome through the symbiosome membrane. It is still unclear how inorganic carbon (C_i) is taken up from seawater by the bacteriocytes. However, two C_i uptake mechanisms are proposed: (A) C_i uptake by a bicarbonate transporter on the cell membrane; and (B) C_i uptake by membrane-associated CA. Because these mechanisms were not detected in the present study, they are marked with question marks.

cotransporter) was identified in a squid giant axon and shown to be strongly expressed in the gill, and it is thought that the transporter plays a role in HCO_3^- uptake by squid gill tissue (Piermarini et al., 2007). If *Calyptogena* clams have a bicarbonate transporter, they could take up HCO_3^- from seawater (Fig. 8A). Then, the intercellular HCO_3^- would be converted to CO_2 by the cytosolic CAs and supplied to symbionts in the symbiosome. Membrane-associated CA was also shown to play a role in C_i uptake in green algae (Tsuzuki and Miyachi, 1989), in giant clam–zooxanthellae symbiosis (Baillie and Yellowlees, 1998) and in photosymbiotic cnidarians (Furla et al., 2000). Membrane-associated CA converts HCO_3^- to CO_2 in seawater, and the latter permeates the cell membrane (Fig. 8B). After this process, there may be two different pathways: (1) the CO_2 may immediately permeate through the symbiosome membrane and be consumed by symbionts; or (2) it may be converted to reach equilibrium by cytosolic CAs. In the former pathway, CA is not necessary and therefore this pathway is unlikely because CAs are abundant in the cytoplasm (Figs 5–7). In the latter pathway, if the symbiont does not immediately consume the CO_2 from the host cytoplasm, the cytosolic CAs convert it to reach C_i equilibrium in the cytoplasm of bacteriocytes. And this process makes it possible to store a C_i pool in the cytoplasm. While the membrane is impermeable to HCO_3^-, CO_2 in equilibrium in the cytoplasm passes through the symbiosome membrane and is consumed by symbionts, which generates a gradient of C_i concentration between the inside milieu of the symbiosome and the cytoplasm of the bacteriocyte (Fig. 8B). However, neither the bicarbonate transporter nor a membrane-associated CA has been found in *Calyptogena* clams, and the mechanism of C_i uptake remains to be resolved in future studies. In *C. okutanii*, once HCO_3^- and/or CO_2 is taken up from environmental seawater to the cytoplasm, CokCAg1 and CokCAg2 likely catalyze the interconversion of HCO_3^- and CO_2 in the cytoplasm and facilitate C_i transport to the symbiosome, where thioautotrophic symbionts are harbored (Fig. 8). In the symbionts, CO_2 is converted to reach C_i equilibrium, and a C_i pool is made by symbiont CA like host CA, or fixed by RubisCO (Fig. 8).

While CA has been considered to participate in C_i uptake and its supply to symbionts in chemoautotrophic bivalves (Kochevar and Childress, 1996), the mechanism underlying these processes has remained unclear. The prominent, specific expression of CA in the bacteriocyte of *C. okutanii* together with the results of comparative analysis of CA protein expressed in the gill tissue of chemosynthetic bivalves suggest that C_i transport to the symbionts in the cytoplasm of the bacteriocyte is the limiting process of C_i transport in the thioautotrophic symbiont–bivalve symbiosis, which must be facilitated by cytoplasmic CA (Fig. 8). More detailed analysis of C_i transport processes, such as C_i transport from seawater to the cytoplasm of the bacteriocyte, the C_i uptake mechanism by the symbiont and the C_i supply process to RubisCO, are necessary to understand the overall C_i supply system in chemoautotrophic symbioses.

LIST OF ABBREVIATIONS

ASW	artificial seawater
CA	carbonic anhydrase
CBB	Coomassie Brilliant Blue R-250
C_i	inorganic carbon
CokCAg1 and CokCAg2	CA in gill tissue of *C. okutanii*
CokCAmab1	anti-CA mouse monoclonal antibody reacting with both CokCAg1 and CokCAg2
CokCAmab2	anti-CA mouse monoclonal antibody reacting with CAs of *Calyptogena* species and *Bathymodiolus* species
EST	expression sequence tag
FSW	0.22 μm-filtered seawater
LC-MS/MS	liquid chromatography electronspray ionization tandem mass spectrometry
P_{CO_2}	partial pressure of CO_2
PFA	paraformaldehyde
ΣCO_2	total CO_2

ACKNOWLEDGEMENTS

We thank Dr T. Fukuba of JAMSTEC for unpublished environmental pH data from around Hatsushima Island, in Sagami Bay, Japan. We are grateful to the captains and crew of the R/V *Natsushima* and R/V *Yokosuka* and to the operation teams of the DSRV *Shinkai 6500* and ROV *Hyper-Dolphin* for sample collection.

AUTHOR CONTRIBUTIONS

Y.H. and T.Y. conceived and designed the experiments. Y.H. performed all experiments; Y.H., T.M. and T.Y. analyzed all data; Y.N. and M.N. contributed to the monoclonal antibody study; Y.T. provided advice on EST analysis; S.S. contributed to LC-MS/MS analysis; K.U. carried out the immunoelectron microscopy; T.T. provided advice concerning the measurement of CA activity and inorganic carbon concentration in deep-sea environments; H.H. and K.T. contributed to the sampling of *C. nautilei* and the discussion in this paper. All the data and analyzed results were examined carefully and discussed by all authors. The final manuscript was read and approved by all authors.

COMPETING INTERESTS

No competing interests declared.

FUNDING

This work was supported by KAKENHI of the Japan Society for the Promotion of Science [grant number 24570252 to T.Y.]. Deposited in PMC for immediate release.

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